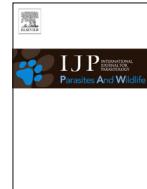




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Negative effects of *Kudoa islandica* n. sp. (Myxosporea: Kudoidae) on aquaculture and wild fisheries in Iceland



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ABSTRACT

In the early 2000s, experimental rearing of spotted wolffish, *Anarhichas minor*, was started in Iceland. Health surveillance, carried out at regular intervals during the rearing period, revealed persistent and highly prevalent *Kudoa* infections of fish muscles which caused great financial losses due to post mortem myoliquefaction. In addition, during the traditional process of drying and smoking wild Atlantic lumpfish, *Cyclopterus lumpus*, the muscles from some fish almost completely disappear and the fish have to be discarded.

To describe the etiological agent responsible for these conditions, spotted wolffish, Atlantic wolffish *Anarhichas lupus*, northern wolffish *Anarhichas denticulatus* and Atlantic lumpfish were caught off the Icelandic coast and examined for the presence of *Kudoa*. We describe a novel myxosporean, *Kudoa islandica* n. sp., using morphological and molecular data, and show with histopathology that it causes extensive myoliquefaction in three different wild fish hosts, which all are commercially valuable species in Iceland. Although some spore dimensions varied significantly between fish species, the molecular analyses showed that the same parasite was responsible for infection in all fish. The northern wolffish was not found to be infected. Although robustly placed in the *Kudoa* clade in phylogenetic analyses, *K. islandica* was phylogenetically distinct from other kudoids.

A single myxosporean, *K. islandica*, is responsible for the infections in the somatic muscles of lumpfish and wolffish, causing extensive post mortem myoliquefaction. This myxosporean is likely to infect other fish species and it is important to study its life cycle in order to evaluate any threat to salmonid culture via the use of lumpfish as a biocontrol for sea lice.

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1. Introduction

Myxosporeans are a diverse group of parasites commonly found infecting fish. Although the life cycles of many species are not described, known life cycles require an alternate host which typically is an annelid worm. To date 95 nominal species of the myxosporean genus *Kudoa* (Kudoidae) have been described, presently all with unknown life cycles. Most commonly kudoids are histozoic in skeletal muscles of fish (Moran et al., 1999a; Lom and Dykova, 2006; Eiras et al., 2014) but have also been found to infect other organs such as brain, heart, gills, kidney, gall bladder, ovary and intestines (Egusa, 1986; Sandeep et al., 1986; Sarkar and Ghosh, 1991; Yurakhno, 1991; Blaylock et al., 2004; Yurakhno et al., 2007; Mansour et al., 2013). Furthermore, at least

one *Kudoa* sp. has been found infecting the musculature of a non-piscine host, the giant octopus *Paroctopus dofleini* (Yokoyama and Masuda, 2001). Although most *Kudoa* species are described from a single host, several have been identified in numerous fish species from different families, such as *Kudoa thrysites* (with at least 38 different hosts), *Kudoa nova* (20 hosts) and *Kudoa iwatai* (19 hosts) (Burger and Adlard, 2011). While kudoids are generally not considered highly pathogenic to the host, some species have caused significant detrimental effects on both commercial fisheries and aquaculture, due to the unsightly muscle cysts they form and more importantly the post mortem myoliquefaction some species cause, commonly known as “soft flesh”, which greatly reduces the market value of the fillets (Langdon, 1991; Alvarez-Pellitero and Sitjà-Bobadilla, 1993; Moran et al., 1999a). In addition, cysts of *Kudoa septempunctata* from farmed fish in Japan and Korea have been strongly linked with numerous cases of food poisoning in humans (Matsukane et al. 2010; Kawai et al. 2012; Iwashita

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et al., 2013). Therefore, the production of *Kudoa*-free fish products from aquaculture ventures has become a priority.

Atlantic wolffish *Anarhichas lupus* L. 1758, spotted wolffish *Anarhichas minor* Olafsen, 1772 and Atlantic lumpfish *Cyclopterus lumpus* L. 1758 are all commercially important species in Iceland, the mean annual catch over the last 10 years being around 14,000 tn, 2500 tn and 5500 tn, respectively (Anonymous, 2012). The wolffish species are considered desirable products because of their rich and tasty fillet but also their unusual and popular skin, which is used for making designer wear. The northern wolffish *Anarhichas denticulatus* is common in sea water around Iceland, especially off the NW coast, but is not considered a commercially valuable species due to the unusual texture of its flesh. Lumpfish are targeted during a coastal spring fisheries as their valuable eggs are harvested for use as a caviar substitute. Traditionally, female lumpfish are dried and the fillets of male fish are smoked, and processed for human consumption. Recently, new markets have opened for lumpfish muscle and presently most of it is exported to Asia.

Knowledge of *Kudoa* infections in wild Icelandic fish are very scarce. The only report of such infections are in a local fisheries magazine from 1986, where infections in lumpfish muscle are mentioned (Thorsteinsson, 1986) and a report on the healthiness of seafood from 1999 where *Kudoa* sp. is briefly mentioned causing spoilage of fillets of lumpfish and Atlantic wolffish (Bjarnason et al., 1999). No data existed on *Kudoa* in the spotted wolffish until 2003 when an experimental rearing of this fish species was carried out in land based tanks in Iceland. The major problems experienced during culture and harvest were persistent *Kudoa* infections in muscles of the spotted wolffish. No data exists on *Kudoa* infections in northern wolffish.

The aim of the present study was to identify and describe the kudoid myxosporean responsible for the problems experienced during the rearing of the spotted wolffish and to analyse the detrimental effect of the parasite on the farmed fish. In addition, to identify the *Kudoa* causing spoilage of fillets of lumpfish and Atlantic wolffish. Although of no commercial value, the northern wolffish is closely related to the other two wolffish species so was included in the study.

2. Materials and methods

2.1. Fish studied

The experimental rearing of spotted wolffish in Iceland was carried out in shallow raceways in a land-based facility. The broodstock consisted of wild fish which were sampled in June 2002. In the beginning, while adopting to dry food pellets, they were fed on homogenised fish pulp (mostly herring and capelin). The first generation of farmed juvenile fish hatched in February 2003. They were kept separate from the broodfish for the entire rearing period and fed on commercially formulated feed. The intake seawater for the farm, which was taken at 30 m depth from a nearby fjord, was unfiltered in the case of the broodfish but filtered through a sand filter for the farmed juveniles.

At regular intervals, fish were sent to the Fish Disease Laboratory at the Institute for Experimental Pathology at Keldur for thorough examination with regards to infectious diseases. Broodfish (length 60–80 cm – corresponding to age 5–12 year according to Gunnarsson (2010)) were examined in November 2002 (2 fish), March 2003 (2 fish), November 2003 (4 fish) and January 2004 (4 fish). Examination of the 1st generation of the farmed fish was performed twice in 2004; January (2 fish; age = 11 months – length 18–20 cm) and April (16 fish; age 14 months – 22–28 cm) and three times in 2005; April (4 fish; age 26 months – 34–35 cm), May (4 fish; age 27 months – 34–36 cm) and June (4 fish, age 28 months – 37–41 cm).

In February 2013, wild spotted wolffish (length 65–103 cm), Atlantic wolffish (62–89 cm) and Atlantic lumpfish (41–48 cm), 5 fish of each species, were caught in Bay Faxaflói off the west coast of Iceland and examined for the presence of *Kudoa* spp. In addition, in July 2013, ten northern wolffish (length, 65–105 cm), were caught off the NW coast of Iceland and examined for *Kudoa* infections.

2.2. Examination of fresh fish and sampling methods

Skeletal muscles of all fish, farmed and wild, were examined for the presence of *Kudoa* sp. Three slices of muscles, approximately 4 cm wide and 8 cm long, from the anterior-, mid- and posterior parts of the fish fillets, were thoroughly searched under a stereomicroscope for the presence of plasmodia/pseudocysts. If detected, they were removed from the fillet and placed on a microscope slide and examined at 400 \times magnification, to confirm the presence of *Kudoa* spores. If no plasmodia were detected, the muscle slices were homogenised in PBS using a pestle and mortar. The homogenised muscle was sieved (500 μ m) and the fluid centrifuged at 1500 g for 5 min and the pellet microscopically screened for the presence of *Kudoa* spores. Infection intensity was determined as follows: very light = no pseudocysts seen but spores detected from centrifuged muscle homogenate; light = 1–20 pseudocysts detected in any of the muscle slices; moderate = pseudocysts detected in all muscle slices, total number 20–50; severe = pseudocysts detected in all muscle slices, total number >50.

The size of 10 pseudocysts from each fish species (except the wild spotted wolffish) were measured and photographed using a stereomicroscope. Morphological characteristics of 50 fresh spores from each fish species (except for spore length: n = 30 and length of polar filaments: n = 10) were measured following the recommendations of Lom and Arthur (1989) and Adlard et al. (2005).

Pieces of fresh trunk muscle from all examined fish were fixed in 10% buffered formalin at approximately 24 h p.m. In addition, moderately or heavily infected muscle samples from two fish of all species (wild Atlantic wolffish and lumpfish, farmed spotted wolffish), were kept at 4 °C until fixed for histopathological examination, at 48 h p.m. To examine whether freezing affected the p.m. proteolysis of muscles, portions of muscles from one heavily infected wild Atlantic lumpfish were frozen at 48 h p.m. then thawed and stored at 4 °C for 24 h and 120 h before being fixed in 10% buffered formalin.

Air dried smears from infected muscles were fixed in methanol, stained with Giemsa and mounted and used as reference material.

2.3. Histology

Formalin fixed muscle samples were embedded in paraffin wax, sectioned (4 μ m) and stained with Giemsa and Haematoxylin and Eosin (HE). The parasite and associated histological changes were examined using a compound microscope.

2.4. Scanning electron microscopy

For SEM examination, whole plasmodia were removed from infected muscle from Atlantic wolffish and placed in a tube containing PBS and gently disrupted to liberate the spores. Tubes were then centrifuged at 1500 g for 5 min, the supernatant removed and the pellet fixed in 2.5% glutaraldehyde for 4 h. After fixation, the spores were prepared for SEM and viewed following the methods described by Kristmundsson and Freeman (2013). In brief, washed spores were syringed onto a polycarbonate membrane, fixed with 1% osmium tetroxide and dehydrated through an ethanol series. Membranes were dried, mounted on stubs, sputter-coated with gold and viewed.

2.5. Molecular analyses

Kudoa-infected muscle samples from three individuals of wild Atlantic lumpfish and Atlantic wolffish and two wild spotted wolffish as well as muscle samples from three visibly uninfected northern wolffish, were used for the molecular work. Either whole plasmodia (Atlantic wolffish and lumpfish), a pellet from the centrifuged sieved muscle material (spotted wolffish) or small bits of muscle (northern wolffish), were fixed in 95% ethanol for molecular analysis. No molecular work was performed on the farmed spotted wolffish as no samples were available.

Total DNA was extracted using a GeneMATRIX kit (EURx Poland) following the tissue protocol. Parasite small subunit ribosomal DNA (SSU rDNA) was amplified using the myxosporean PCR primers and methodology described by Freeman et al. (2008). To confirm the presence of the parasite in light infections or its absence in those specimens where no spores were seen during microscopy, *Kudoa* specific primers were designed from an alignment of kudoid taxa using CLUSTAL X (Thompson et al., 1997). The primer pair Kud-80f 5' actgcgaagcgctgat Kud-730r 5' aggccacacctcgcaagtgc utilised the same conditions as the above myxosporean primers and were designed to amplify approximately 650 bp of phylogenetically informative SSU rDNA, including the variable V4 region, for the majority of *Kudoa* spp. In addition parasite large subunit ribosomal DNA (LSU rDNA) was amplified using the primers and conditions described by van der Auwera et al., 1994 and Bartošová et al. (2009). In brief, the primer pairs NLF-184/NLR-1270 and NLF-Kud/NLR-3284, were used to amplify two sections of the LSU spanning domains 1–5 and 5–11, respectively. To increase detection sensitivity for samples with low parasite template concentrations, an additional *Kudoa*-specific primer pair, Kud-1f 5' tcaaacttccaaccggtag and Kud-1r 5' ttgcgtatgttcaggct, were designed to amplify a 750 bp section of the LSU spanning domains 5–7b, and bridging any ambiguous overlap region between the initial non-specific LSU sequence reads. A further forward primer Kud-2f 5' aagcctgaaagagcatcgca was used with the reverse primer NLR-3284 to confirm the long reads obtained from the initial amplification of NLF-Kud/NLR-3284. Novel LSU primers were used with the following PCR conditions; initial denaturing for 4 min at 95 °C followed by 35 cycles of: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, with a terminal extension at 72 °C for 7 min.

All PCRs were completed in triplicate and PCR products of the expected sizes were recovered using a GeneMATRIX PCR products extraction kit (EURx Poland). Sequencing reactions were performed using BigDyeTM Terminator cycle sequencing chemistry utilising the same oligonucleotide primers that were used for the original PCRs. DNA sequencing was performed in both forward and reverse directions for all PCR products and nucleotide BLAST searches performed for each sequence read to confirm a kudoid origin (Zhang et al., 2000). The contiguous sequences were obtained manually using CLUSTAL X and BioEdit (Thompson et al., 1997; Hall, 1999). CLUSTAL X was used for the initial SSU rDNA sequence alignments of 16 carefully chosen kudoid taxa, taken from BLAST search results and preliminary experimental alignments which were designed to cover a sufficient phylogenetic range of kudoid taxa whilst minimising long-branch attraction artefacts, by not including other kudoids known to consistently occupy single unresolved branches in previous studies. The final SSU rDNA alignment was manually edited using the BioEdit sequence alignment editor and contained 1610 characters of which 1268 were informative sites. Phylogenetic analyses were performed using the maximum likelihood methodology in PhyML (Guindon et al., 2010) with the general time-reversible substitution model selected and 1000 bootstrap repeats.

2.6. Statistical analyses

Statistical analyses of spore measurements were performed in Rstudio (version 0.98.501) using Bartlett Test of homogeneity of variances, Tukey multiple comparisons of means and anova.

3. Results

3.1. Progress and prevalence of infections

Kudoa infections were not detected in farmed spotted wolffish broodfish, examined in November 2002 and March 2003, by stereo- and microscopic examination of fresh material and histological sections. In November 2003 two of four fish examined had severe infections while the remaining two were uninfected. At that time, the infections were characterised by significant numbers of small plasmodia containing developing spores with few mature spores detected. In January 2004, two of four fish were infected, one severely and one lightly while no infections were detected in two fish. The one with the severe infections had a mixture of fully mature plasmodia (with mature spores) and smaller ones containing developing spores.

The first two fish of the 1st generation of farmed juvenile spotted wolffish, examined in January 2004, were lightly infected with *Kudoa* and similarly, all 16 fish examined 3 months later. The relatively few plasmodia observed in these fish were small and contained mostly immature, developing spores. The fish examined in April, May and June 2005, were all lightly or moderately infected, with a mixture of mature and developing spores.

Kudoa infections were not detected in the northern wolffish while two of five of the wild spotted wolffish and all five Atlantic wolffish and lumpfish were found infected with *Kudoa*. Both infected spotted wolffish, had very light infections and no plasmodia were detected. Light to moderate infections were observed in the Atlantic wolffish with plasmodia easily observed in the fillets using a stereo microscope. The lumpfish were most heavily infected, two fish had moderate infections and three had severe infections where, in some cases, large proportions of the trunk muscle were replaced with plasmodia of *Kudoa* (Figs. 1, 2A).

3.2. Description of *Kudoa islandica* n. sp

3.2.1. The plasmodia

Fresh plasmodia appear whitish, tubular (size up to 11 mm × 1.4 mm), with a narrow tip at one end (Fig. 1A and B). They are located inside muscle fibres and encased by a relatively thin membrane (Fig. 2B–E) separating the spores from the host muscle. Infections are either single (Fig. 2B), i.e. one plasmodium developing inside a single muscle fibre, or multiple where two or more, in many cases numerous, plasmodia develop inside the same muscle cell (Fig. 2C–E). When fully developed, the sarcoplasm of the greatly enlarged infected muscle cell is virtually fully substituted by plasmodia, with the compressed remnants of the muscle fibre and in some cases adjacent fibres, firmly encasing the parasitic plasmodia forming a pseudocyst (Moran et al., 1999b) which could easily be removed undamaged from the muscle with fine forceps.

3.2.2. The spores

The spores are square shaped with rounded peripheral edges in apical view and garlic shaped in the lateral view. The spore contains four pyriform and equally sized polar capsules containing 3 coils of relatively short polar filaments (Figs. 3 and 4). Scanning electron microscopy revealed the presence of four prominent apical projections as well as irregularly situated cytoplasmic

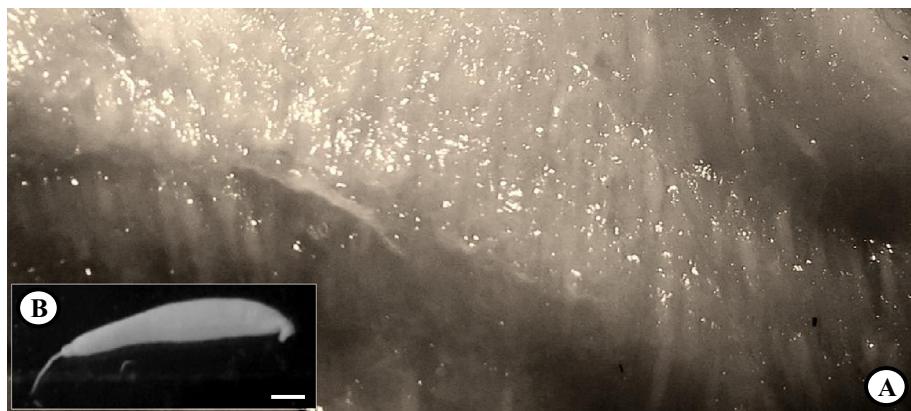


Fig. 1. (A) Fresh fillet from lumpfish, *Cyclopterus lumpus*, packed with plasmodia. (B) Freshly isolated plasmodium. Scale bars: (A) = 10 mm; (B) = 1 mm.

projections of various lengths at the apical side of the spore. Sutural lines between the valves were fairly fine, but clearly visible and sometimes curved along their length (Fig. 4). Detailed measurements of spores from all fish hosts are shown in Table 1. The range of dimensions of all isolates of *K. islandica* spores were: Width, 6.5 – 9.5; thickness, 5.0 – 8.0 µm; length, 4.1 – 6.8 µm; suture width, 4.4 – 7.8 µm; length of polar capsules, 1.4 – 2.5 µm; width of polar capsules, 1.2 – 1.9 µm; length of polar filaments, 3.6 – 5.2 µm. Spore measurements of *K. islandica* isolates, from the three different host, were statistical different in all cases with regards to width/thickness, length and suture width. Except for spore width/thickness between the two wolffish species ($p = 0.02$), the significance level was $p < 0.01$.

3.2.3. Histopathology

No inflammation, fibrosis or any other host response was detected in relation with infections. In muscles fixed at approx. 24 h p.m., liquefactive necrosis was not commonly detected in association with infections. Occasionally, the thin plasmodial membrane was broken exposing the spores to muscular tissue leading to focal muscular necrosis in the close vicinity of the liberated spores (Figs. 5A, B).

In muscles fixed approx. 48 h p.m. extensive myoliquefaction was commonly observed. The level of muscular liquefaction was dependent upon the number of free spores and their distribution inside the muscle, as it was in all cases limited to the presence of spores (Fig. 5C–E).

Freezing the muscle did not impede the proteolytic activity of the parasite. No degradation occurred whilst the muscle was frozen but when thawed, the process of myoliquefaction resumed, with a gradual increasing number of ruptured plasmodia detected followed by an increasing number of free spores in the tissue resulting in more extensive liquefactive necrosis. At 120 h after thawing (corresponding to 168 h p.m.), the muscles were very loosely bound and large empty areas of the muscular tissue were completely necrotised and sloughed away. However, the muscle cells that were not affected by the parasite were still surprisingly undamaged, indicating a minor contribution of autolysis to the degradation of the muscular tissue.

3.2.4. Molecular analyses

Contiguous SSU rDNA sequences were successfully obtained from three individual fish (two for spotted wolffish) from each of the three infected fish species. All SSU rDNA sequences were 100% identical over 1735 bp of data. LSU rDNA sequences were also successfully amplified from three infected lumpfish and Atlantic wolffish and the two spotted wolffish found infected. However, parasite DNA was only recovered from the spotted wolffish using the specific *Kudoa* LSU primers (Kud-1f/Kud-1r). No parasite DNA was

amplified from the muscle of the northern wolffish in either SSU or LSU PCR. LSU sequences from the Atlantic lumpfish and the Atlantic wolffish were 100% identical over 3339 bp. LSU sequences from the spotted wolffish were also 100% identical to the other sequences but over a region of 765 bp, demonstrating that the same species, *K. islandica* n. sp., was responsible for infection in all cases. Sequence have been submitted to GenBank with the accession number KJ451388 (SSU) and KJ857071-2 (LSU). BLAST searches of the new sequences revealed a 94–96% similarity with other kudoid taxa for the SSU rDNA and 88–93% similarity with numerous *Kudoa* spp. for the LSU rDNA. Phylogenetic analysis of the SSU rDNA robustly placed *K. islandica* within the *Kudoa* clade; however, it was not well supported in any of the existing clades (Fig. 6).

3.3. Taxonomic summary

Phylum: Cnidaria Hatchek 1888

Unranked subphylum: Myxozoa Grassé 1970

Class: Myxosporea Bütschli 1881

Order: Multivalvulida Shulman 1959

Family: Kudoidae Meglitsch 1960

Genus: *Kudoa* Meglitsch 1947

Species: *islandica*

Type hosts: Atlantic lumpfish *C. lumpus* L. 1758

Other hosts: Atlantic wolffish *A. lupus* L. 1758, spotted wolffish *A. minor* Olafsen, 1772.

Location: Icelandic waters.

Site of infection: Intracellular in skeletal muscles.

Etymology: The specific name *islandica* refers to the type locality.

Type material: Two Giemsa-stained histological sections and one stained wet mount slide have been submitted to the collections of the Natural History Museum, London, and assigned the accession numbers: NHMUK 2014,1 (holotype: muscle-histological section, Atlantic lumpfish); NHMUK 2014,2 (Paratype: muscle-imprint, Atlantic wolffish) (NHMUK 2014,3 Paratype: muscle-histological section, spotted wolffish). DNA sequence data has been submitted to GenBank with the accession number KJ451388 (SSU) and KJ857071-2 (LSU).

Nomenclatural acts: The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature (ICZN), and hence the new name contained herein is available under that Code from the electronic edition of this article. This published work and the nomenclatural act it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifier) for this publication is: urn:lsid:zoobank.org:act:1B81F431-98AD-4418-91CC-9D9975CABAE1.

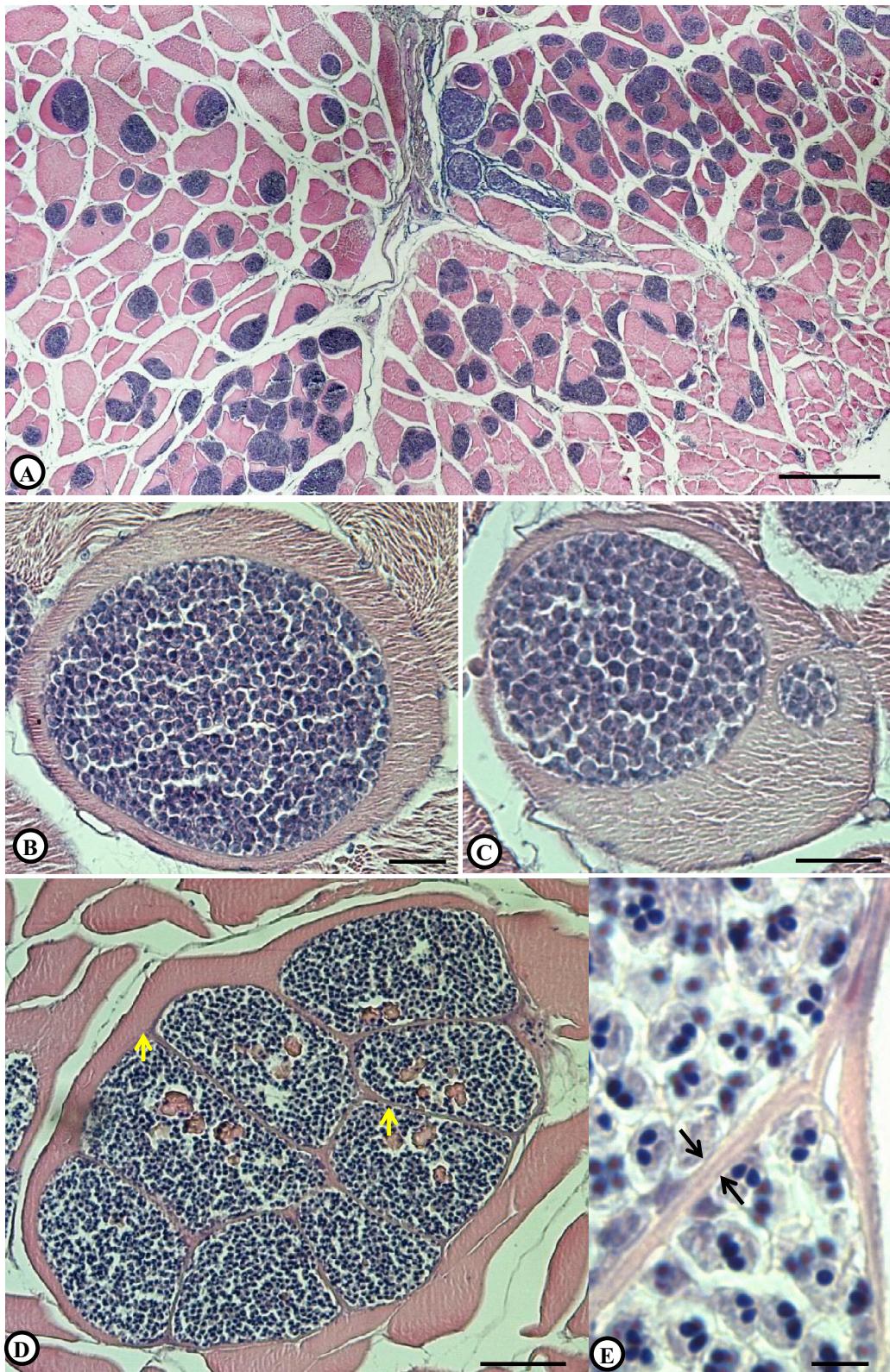


Fig. 2. (A) Stained histological section of a lumpfish muscle showing a considerable portion of the muscle fibres substituted with *Kudoa islandica* n. sp. plasmodia. (B) A single infection. (C) A double infection. (D) Multiple infection; numerous plasmodia developing inside a single muscle fibre, separated from each other and the muscle tissue with a thin membrane (arrows). (E) Plasmodial membranes separating two plasmodia (arrows). Inside each plasmodium are numerous mature *Kudoa* spores. Scale bars: (A) = 300 µm (B) and (C) = 25 µm; (D) = 150 µm; (E) = 5 µm.

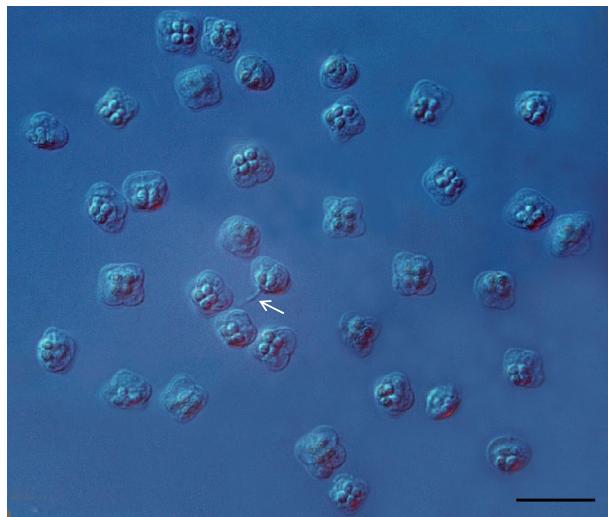


Fig. 3. Fresh mature spores of *Kudoa islandica* n. sp. as seen in fresh squash preparations from muscular tissue of Atlantic wolffish, *Anarhichas lupus*. Note the protruding polar filament of one of the spores (arrow). Nomarski differential interference contrast. Scale bar = 10 µm.

4. Discussion

K. islandica n. sp. is the first kudoid species to be described from Icelandic waters. Morphologically, it has a typical quadrate form but only has a 95% genetic similarity in the SSU rDNA and an 88–93% similarity in the LSU rDNA to other known *Kudoa* species. Although *K. islandica* is robustly placed with other kudoid taxa in phylogenetic analyses, it is not closely related to other described *Kudoa* spp. and occupies an unresolved position in both SSU rDNA and LSU rDNA (data not shown) phylogenetic analyses. However, in SSU rDNA analyses it is reproducibly placed in a clade containing the type species, *Kudoa clupeidae* and *Kudoa rosenbuschi* that form as a sister clade to the one containing *Kudoa paniformis*. *K. islandica* is most similar in terms of plasmodia type, spore size and morphology to three kudoid species (Table 1): *K. paniformis*, *Kudoa miniauriculata* and *K. rosenbuschi* (Eiras et al., 2014), however, existing DNA data for all three species exclude them as being conspecific. Although statistically significant differences were observed in spore morphology between host fish species in this study, parasite DNA sequences were identical from all host fish, confirming that the same parasite was infecting all fish examined. Significant morphometric differences between fish hosts for the same species of *Kudoa* have been observed before (Blaylock et al., 2004; Burger and Adlard, 2011).

Infections are intracellular in muscle fibres, which is a common site of infection for kudoid myxosporeans. However, although the presence of multiple infections inside a single myofibre has been observed (Lom et al., 1983; Moran et al., 1999c), it is uncommon, and in this respect *K. islandica* is different from most other described species. Lom et al. (1983) detected similar infections patterns in a *Kudoa* sp. from Californian rockfish *Sebastodes paucispinis*. They reported two kinds of pseudocysts, one spindle-shaped located in the longitudinal axis of the muscle fibre and encased directly with the remnants of the fibre, and also pseudocysts containing numerous smaller plasmodia filled with mature spores. The former resembles single infections of *K. islandica* and the latter multiple infections, as commonly observed in our study. Lom et al. (1983) noted that it might suggest two separate species infecting the same host. However, Whitaker and colleagues (1996) who examined the same fish species from the same locality, only found it infected with a single species, *K. miniauriculata*,

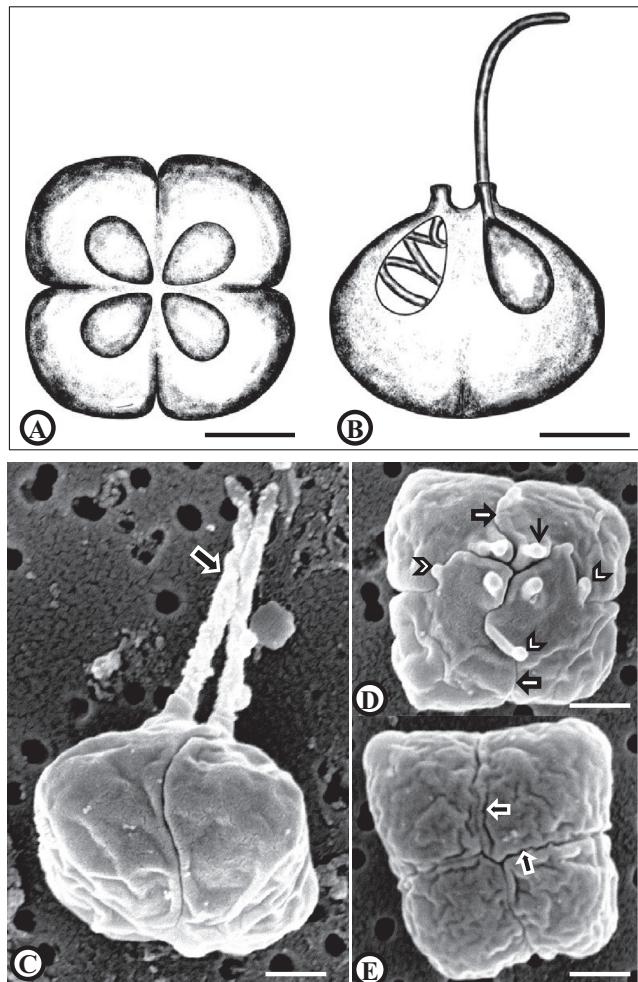


Fig. 4. Line drawings of *Kudoa islandica* n. sp. in apical view (A) and lateral view (B). Scanning electron microscope images of *K. islandica* n. sp. (C–E). Mature spore in lateral view showing extruded polar filaments (arrow) (C). Single spore in apical view (D) showing the sutures of the four valves (broad arrows), the four apical projections (thin arrow) and cytoplasmic projections (arrowhead). Single spore in posterior view (E) showing the suture of the four valves (broad arrows). Scale bars: (A) and (B) = 2 µm; (C), (D) and (E) = 1 µm.

regardless of the appearance or size of the pseudocysts. Similarly, in the present study, although single and multiple infections were present simultaneously in all infected fish, neither spore morphology nor results of DNA sequencing suggested the presence of two separate species.

The presence or absence of a host response varies greatly between *Kudoa* species. In some cases it can be severe, including substantial infiltration of inflammatory cells and granuloma formation, while in others, like *K. islandica*, no host response is detected (Whitaker et al., 1996; Casal et al., 2008; Dyková et al., 2009). It is possible that numerous *Kudoa* species, such as *K. islandica*, remain undetected by the host immune system due to their intracellular location, firmly enveloped by the remnants of the muscle fibre and the surrounding connective network of the endomyium. Egusa (1986) summarised the effect of *Kudoa* infections on living fish. His results demonstrated that even though there were localised pathological changes, no effect on physiology, behaviour or life span of the fish host was observable. Indeed, the *Kudoa* infections did not appear to have any detrimental effect on the cultured spotted wolffish in Iceland, suggesting there is little impact caused by infection with *K. islandica* in live fish. However, in the most severe infections where large parts of the

Table 1
Range and mean value (within parenthesis) of morphological measurements of *Kudoa islandica* n. sp. spores from Atlantic lumpfish, *Cyclopterus lumpus*, Atlantic wolffish, *Anarhichas lupus*, and spotted wolffish, *A. minor* and other morphologically or genetically similar kudooids infecting muscular tissue of fish. Abbreviation: n.a. = no data available, PC = polar capsules.

	Width	Length	Thickness	Suture width	PC length	PC width	Polar filaments	Plasmodia	Morphology size	Fish host	Locality	References
<i>K. islandica</i>	6.5–8.6 (7.4)	4.1–5.1 (4.8)	5.0–6.5 (5.6)	4.4–5.5 (4.9)	1.4–1.9 (1.7)	1.2–1.8 (1.5)	3.6–5.0 (4.3)	Tubular	<i>Cyclopterus lumpus</i>	Iceland	Present study	
<i>K. islandica</i>	8.6–9.4 (8.9)	5.1–6.2 (5.9)	6.2–7.9 (7.2)	5.5–7.5 (6.6)	1.4–2.5 (2.2)	1.2–1.9 (1.5)	3.8–5.1 (4.4)	$\leq 11 \times 1.4$ mm	<i>Anarhichas lupus</i>			
<i>K. islandica</i>	8.6–9.5 (9.0)	5.5–6.8 (6.2)	6.5–8.0 (7.4)	5.8–7.8 (7.0)	1.7–2.4 (2.1)	1.5–1.9 (1.7)	3.9–5.2 (4.5)	<i>Anarhichas minor</i>				
<i>K. paniformis</i>	5.0–6.5 (5.9)	4.5–6.0 (5.0)	6.0–7.0 (6.6)	n.a.	1.9–2.4 (2.0)	1.4–1.9 (1.6)	n.a.	Tubular	<i>Merluccius productus</i>	Canada	Kabata and Whitaker (1981)	
<i>K. miniauriculata</i>	7.0–8.5 (7.9)	5.0–5.9 (5.4)	n.a. (7.9)	n.a.	1.8–2.3 (2.2)	n.a.	n.a.	n.a.	<i>Sebastes paucispinis</i>	USA	Whitaker et al. (1996)	
<i>K. rosenbuschi</i>	n.a. (7.0)	n.a.	n.a. (7.0)	n.a.	n.a. (2.5)	n.a.	n.a.	n.a.	<i>Merluccius</i> spp.	Argentina	Abollo et al. (2005)	
<i>K. clupeidae</i>	6.3–7.5 (6.4)	4.0–5.3 (5.1)	n.a.	n.a.	1.5–2.6 (2.0)	–(1.0)	n.a.	Tubular	<i>Clupea harengus</i>	USA	Eiras et al. (2014)	
								Length 5 mm				

muscle are substituted by *Kudoa* pseudocysts, as observed in wild lumpfish in the present study, it is hard to imagine that such infections do not affect the functionality of the muscle to some extent.

K. islandica is similar to other *Kudoa* species, such as *K. iwatai*, *K. nova* and *K. thysrites* (Burger and Adlard, 2011), as it is nonspecific with regards to host fish, infecting unrelated fish such as wolffishes (Order: Perciformes, Family: Anarhichadidae) and lumpfish (Order: Scorpaeniformes, Family: Cyclopteridae). This suggests that habitat or diet could play a role in *K. islandica* infections dynamics or transmission cycles. Atlantic and spotted wolffish are benthic species which prefer a sandy or muddy bottom. The habitat of lumpfish depends on age and season; mature fish move inshore in the spring and early summer to spawn in shallower coastal waters but return to deeper waters offshore in late summer and early autumn, while the juveniles spend their first year of life among weed clumps in bays and fjords. A considerable portion of the diet of all these three fish species is composed of benthic animals like echinoderms, crustaceans, ctenophores, molluscs, polychaetes and smaller fish (Moring, 1989; Kristinsson et al., 1997; Kristjánsson et al., 1997; Jónsson and Pálsson, 2013). The northern wolffish is, however, considered more pelagic and consequently its diet contains fewer benthic species (Jónsson and Pálsson, 2013). As all known marine myxosporean life cycles include benthic polychaetes as alternate hosts, this could explain the lack of *K. islandica* infections in the northern wolffish. The lack of host specificity of *K. islandica*, and the fact that numerous other fish species utilise a similar habitat as the three known hosts; it would seem likely that other fish species are susceptible to infection by this parasite.

The most serious impact of *K. islandica* is the spoilage of fish fillets as it causes extensive post mortem myoliquefactive necrosis. The effect and progress of this is similar in all three fish species examined, shortly after fish death the thin plasmoidal membrane degenerates exposing the spores to the muscular tissue enveloping the parasites. The presence of the spores causes this muscular envelope to necrotise with a subsequent liberation of numerous spores into the surrounding muscular tissue. The presence of large white pseudocysts also reduces the marketability of the product.

Myoliquefaction and the presence of unsightly cysts in fish fillets due to *Kudoa* infections has been known to cause significant damage to products of commercially valuable fish species for decades (Moran et al., 1999a). Although poorly studied, the cause of post mortem myoliquefaction has long been thought to be due to proteolytic enzymes released by the parasite (Moran et al., 1999a). Funk et al. (2008) characterised a protease from *K. thysrites* responsible for myoliquefaction in Atlantic salmon, *Salmo salar*, and found it to be pH dependant. Following death of the Atlantic salmon, the pH decreases due to anaerobic glycolysis, which generates lactic acid. This decrease apparently triggers a processing of the enzyme, which is stable in live fish, resulting in post mortem muscle proteolysis. Therefore, reducing the amount of glycogen in the muscle fish, prior to harvesting, was considered a possible way of reducing the post mortem effect of *K. thysrites* (Funk et al., 2008). However, due to potential interspecies differences of both fish and *Kudoa* species, the results from that study cannot necessarily be extrapolated to all *Kudoa* species, and also may not be easy to implement.

Moran et al. (1999a) published a review of the known species from the genus *Kudoa*. At that time 44 species had been identified, 27 of which infect somatic muscles of various fish species and nine of those were known to cause post mortem myoliquefaction with numerous others developing into large visual and unsightly cysts affecting the value of the fillets. Since this report, a further 51 *Kudoa* species have been described, as well as new hosts for formerly described species, some of which (*Kudoa camarguensis*, *Kudoa megacapsula* and *Kudoa aequidens*) considerably reduce the

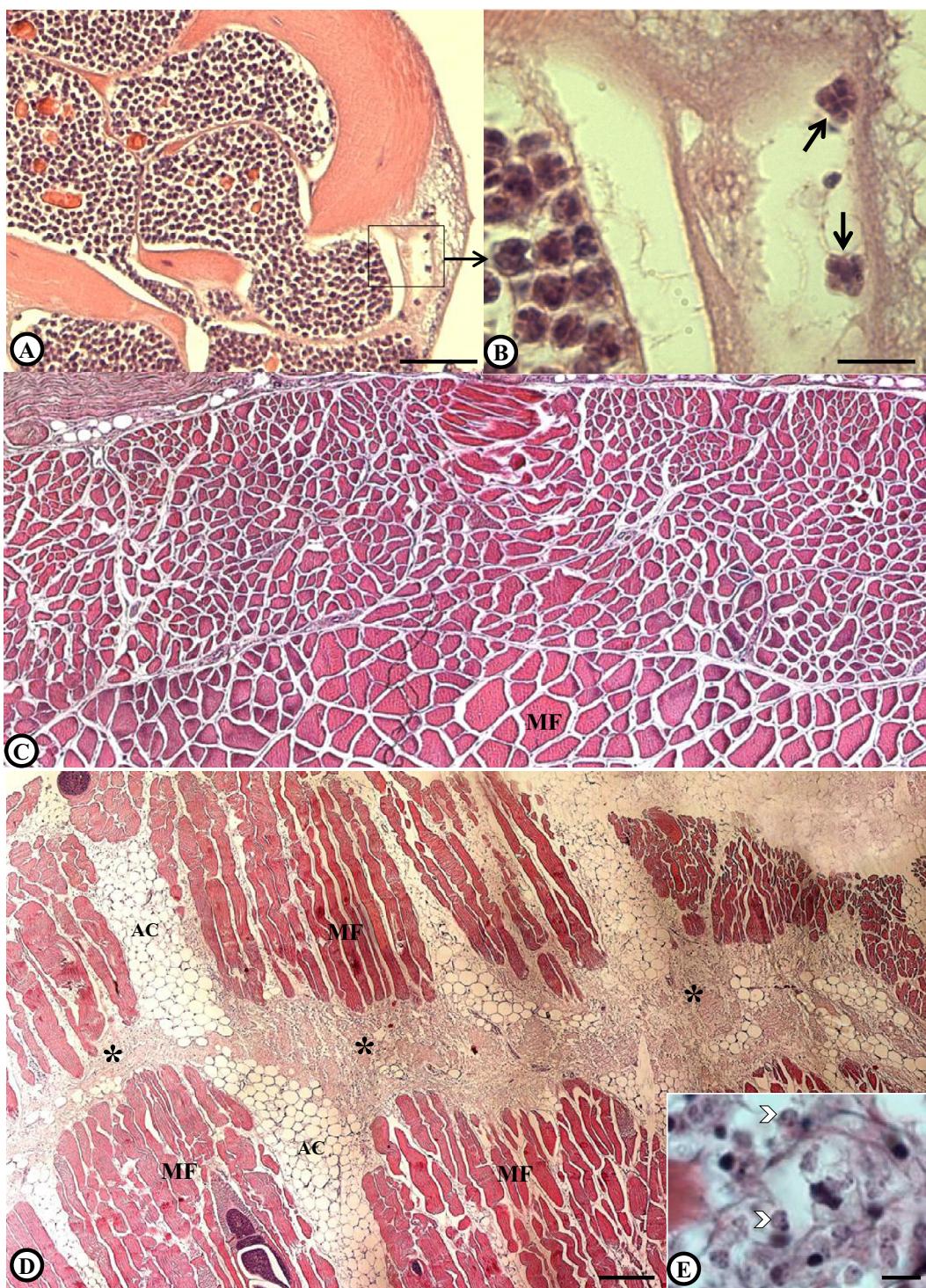


Fig. 5. (A) and (B) Muscle section from lumpfish, *Cyclopterus lumpus*, fixed 24 h post mortem. (A) Ruptured *Kudoa* plasmodia with subsequent liberation of mature spores causing focal necrosis of the muscle fibre enveloping the plasmodium. (B) Higher magnification showing liberated spores (arrows) and a focal necrosis in the vicinity of the spores (asterisk). (C) Muscle section of an uninfected fish at approx. 48 h p.m. (D) Section of muscle of a heavily infected fish at approximately 48 h p.m. showing extensive myolytic necrosis (asterisk). (E) A close up of the affected area showing numerous *Kudoa* spores (arrowhead) and the associated liquefactive necrosis. Scale bars: (A) = 50 µm, (B) = 10 µm, (C) and (D) = 200 µm, (E) = 10 µm. Abbreviations: MF = Muscle fibres, AC = Adipocytes.

commercial value of the fish species they infect (Yokoyama and Itoh, 2005; Pampoulie et al., 1999; Casal et al., 2008).

Infections with *K. islandica* appear to be prevalent in wild lumpfish and in at least two of the three wolffish species present in Icelandic waters. Despite a lack of scientific publications on *Kudoa* in Icelandic fish, the knowledge of soft flesh fish has been known

for decades among fishermen and people processing fish. In Iceland, female lumpfish are traditionally dried for human consumption after roe harvesting. It has been noticed over the years that during this process some fish wither and after drying contain numerous white spots and have little muscle compared to other fish. Similarly, male lumpfish fillets are smoked and used as bread

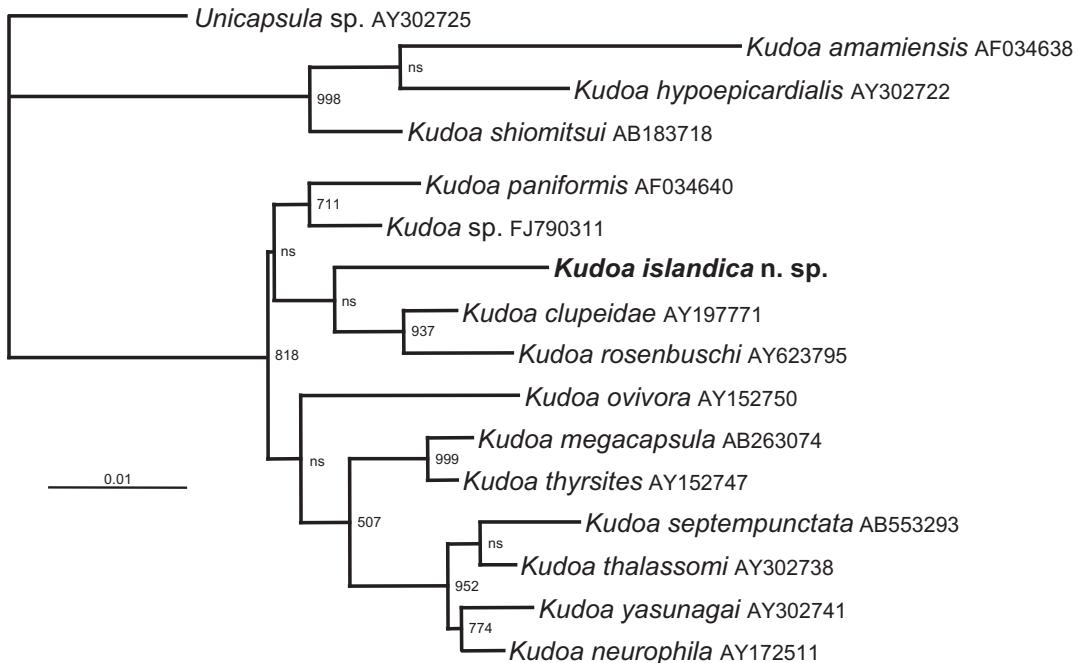


Fig. 6. SSU rDNA maximum likelihood phylogenetic tree of 16 *Kudoa* spp. *Kudoa islandica* is robustly and consistently placed with other *Kudoa* taxa in all analyses, but is not well supported in the clade it is placed in. Numbers at the nodes represent bootstrap support from 1000 samplings, nodes with a support of <50 are considered not supported (ns).

toppings. Prior to smoking, the fillets are soaked in brine to add taste to the product and also to act as a preservative. It has been observed by the processors that fillets having white cysts do not retain the brine and consequently decay. Furthermore, these fillets are known to be undesirable to consume fresh as they disintegrate during cooking, as has been reported with *K. thrysites* (Langdon, 1991). These historical findings are supported by a short report from 1986 where protozoan infections in lumpfish muscle are mentioned as a cause for soft flesh (Thorsteinsson, 1986). Furthermore, infections with an unidentified *Kudoa* sp., causing spoilage of lumpfish flesh, are mentioned in another report from 1999 (Bjarnason et al., 1999). Similar to the lumpfish, muscular infections causing flesh spoilage have also been known in Atlantic wolffish from Iceland for a long time. Infections in Atlantic wolffish with an unidentified *Kudoa* are mentioned in the report from 1999 (Bjarnason et al., 1999). Indeed, among fisherman, soft flesh wolffish have for decades been called “hárasteinbítur” in Icelandic, meaning “hairy wolffish”, referring to the long white pseudocysts present in the fillets (Bjarnason et al., 1999). Unlike lumpfish and the Atlantic wolffish, *Kudoa* infections were unknown from the spotted wolffish until they became a major problem in an experimental farming of this species. Possibly, *Kudoa* infections in the wild spotted wolffish are not as prevalent or as severe as in the Atlantic wolffish and consequently not noticed. However, under semi-intensive rearing conditions there is little doubt that the spotted wolffish is extremely sensitive to *Kudoa* infections. No studies have been made on *Kudoa* infections in the northern wolffish which may be due to the fact that it is not considered suitable for human consumption. It is commonly called the “jelly cat”, due to its jelly-like muscle, and in most cases is thrown back into the sea when caught. Interestingly, these wolffish that are known to have gelatinous musculature were not found to be infected with *Kudoa* in this study and the reason for their unusual muscle consistency remains unknown.

Experimental rearing of spotted wolffish started in Norway in the early 1990s and the first reproducing broodstock was

established in 1993. During the following years, this fish species also received attention in Canada and Chile and latterly in Iceland (Falk-Petersen et al., 1999; Foss et al., 2004). Among farmers and scientists, the spotted wolffish was considered one of the most promising candidates for cold water aquaculture due to its fast growth, its unique farming-friendly behaviour and its robustness with regards to infectious diseases (Falk-Petersen et al., 1999; Espelid, 2002; Foss et al., 2004). The greatest mortality observed in the Norwegian culture of spotted wolffish was due to atypical *Aeromonas salmonicida* infections under stressful conditions, but also to a smaller extent, parasitic infections such as *Ichthyobodo* sp., *Trichodina* sp., *Pleistophora* sp. and *Gyrodactylus* spp. (Espelid, 2002; Foss et al., 2004). In Iceland, there were few pathogens causing problems, other than infections with atypical *A. salmonicida* and *Gyrodactylus anarhichatis* (Kristmundsson et al., 2006). The main difference was that *Kudoa* infections became a serious problem in Iceland but were never reported in other countries farming spotted wolffish. This could be due to the lack of an obligate alternate host, or possibly due to the large number of infected lumpfish found around the Icelandic coast that could act as a reservoir for infection.

As demonstrated in the present paper, *Kudoa* infections gradually increased in the broodstock and similarly in the 1st generation of the farmed juvenile fish. The infections did not appear to affect the fish health and the extent of the problem did not become apparent until the fish were slaughtered. At that time, significant parts of the fish were extensively infected. Post mortem myoliquefaction became evident and many of the fillets proved unfit for human consumption. In 2008, the farming ceased and all fish were slaughtered. There may have been multiple reasons for the termination of wolffish culture in Iceland; however, the negative impact of *K. islandica* certainly played a significant part. Wolffish aquaculture in Norway has also largely ceased; however, this was due to different reasons, notably the early promise of the cod farming attracting the majority of new investment (Foss and Sparboe, 2009).

The detrimental effects of *Kudoa* infections, due to post mortem myoliquefaction and unsightly cysts, have been problematic in the aquaculture industry for decades and in the most severe cases caused the closure of farms (Egusa and Nakajim, 1980; Langdon, 1991; Whitaker and Kent, 1991; Alvarez-Pellitero and Sitjà-Bobadilla 1993; Moran et al., 1999a; Yokoyama et al., 2004). Although, the problems have largely been restricted to fish held in sea pens, where fish are exposed to pathogens in the natural environment, considerable problems have also been experienced in cultured fish in land-based facilities. In addition to *K. islandica* in spotted wolffish farming in Iceland, *K. septempunctata* infections in olive flounder (*Paralichthys olivaceus*) and *Kudoa neurophila* in a striped trumpeter (*Pelates sexlineatus*) hatchery, have been problematic in land-based facilities (Grossel et al., 2003; Grossel et al., 2005; Matsukane et al., 2010; Grabner et al. 2012). This raises questions regarding the life cycle of *Kudoa*. Apart from few known exceptions, such as *Enteromyxum leei* (Estensoro et al., 2010), all known myxosporean life cycles include an alternative host, most commonly an annelid worm. Although infections were not detected in the first broodfish (of wild origin) examined in the spotted wolffish farm, they might have been very lightly infected, similar to the wild ones examined in the present study, and therefore not readily detected. Furthermore, infective stages might have been introduced with the intake seawater, which in case of the broodfish was unfiltered. Unlike the broodfish, the intake seawater for the juveniles was filtered through a sand filter. However, the effectiveness of such filtration as a barrier to infective myxosporean stages is somewhat conflicting. Cobroft and Battaglene (2013) stated that mechanical filtration with a sand filter was not an effective barrier to *K. neurophila* actinospores and variable results had been achieved with mechanical filtration in the prevention of *M. cerebralis* infection in rainbow trout. However, Grossel et al. (2005) detected *K. neurophila* using PCR in all pre-treated water filtrates while all post treated water filtrates were negative. Therefore, it is apparent that sand filtration is not always effective in removing all infective stages from the intake seawater. However, other possibilities, for example the potential existence of a non-annelid alternate host in the rearing environment, such as *Gyrodactylus* spp., cannot be ruled out, as myxosporean hyperparasitism of monogeneans involving basal kudoids has been reported (Freeman and Shinn, 2011). Direct transmission is also a possibility, but infection of *Kudoa* from fish to fish has only been achieved where blood from an infected salmon was injected in the intraperitoneal cavity of a naive salmon (Moran et al., 1999b), suggesting an alternate host is required for natural transmission to occur.

There is no doubt that *K. islandica* causes serious economic losses to wild fisheries in Iceland. To what extent, is likely dependent on the prevalence and intensity of infections (Dawson-Coates et al., 2003). Lumpfish had the most severe infections of the wild fish examined, whether that is due to a higher susceptibility to *Kudoa* infections or greater exposure to the pathogen in the natural environment, remains unknown. However, *Kudoa* infections in lumpfish, and a newly reported microsporidian infection (Freeman et al., 2013), are of concern to the salmon aquaculture industry as lumpfish are increasingly being used as cleaner fish in salmon aquaculture. *K. islandica* is not host specific, infecting fish from different taxonomic orders, and therefore may be able to infect Atlantic salmon and other important fish species.

Due to the recent discovery that *K. septempunctata* is the causative agent of food poisoning in humans (Matsukane et al. 2010; Kawai et al. 2012; Iwashita et al., 2013), the potential effect of muscle-infecting *Kudoa* species upon food safety and public health should be considered. *Kudoa septempunctata* is known to cause food poisoning in humans when accidentally consumed with raw flounder, as sashimi, but has shown to be inactivated when heated to 95 °C for 10 min (Kawai et al. 2012), suggesting that thorough

cooking of fish should eliminate the problem. However, such a high temperature may not be reached internally when certain cooking methods are used and fish is increasing being eaten raw or lightly cooked. Therefore, additional studies should be undertaken to establish additional ways to neutralise problematic *Kudoa* spp. in fish fillets.

Taking into account all 95 nominal *Kudoa* species known, many of which infect the fillets of edible fish, it seems that food poisoning is historically an uncommon complication of eating *Kudoa*-infected fish. The three known fish species that are hosts to *K. islandica* have been consumed by humans for centuries in Iceland without any reports of associated food poisoning. Consequently, it almost certainly has no effect on human health when cooked.

5. Conclusions

A novel myxosporean, *K. islandica* n. sp., is described from Atlantic lumpfish and two species of wolffish from Icelandic waters. Although some spore dimensions varied significantly between fish species, the molecular analyses showed that the same species was responsible for infection in all fish, confirming low host specificity for the parasite. Square-shaped kudoid spores develop in large plasmodia in the somatic musculature, with infections being most severe in lumpfish and moderate in Atlantic wolffish. Spotted wolffish from the wild had very light infections; however, during semi-intensive experimental rearing they became very heavily infected. The northern wolffish was not found to be infected. Infections did not appear to have any detrimental effect on the cultured spotted wolffish in Iceland, suggesting there is little impact caused by infection to live fish. However, *K. islandica* causes extensive post mortem myoliquefaction in all cases and this played a significant role in the closure of spotted wolffish farming in Iceland. It has also caused spoilage of lumpfish products during the traditional process of drying and smoking.

It is important to determine the life cycle for *K. islandica* and other kudoid myxosporeans in order to evaluate their potential danger to salmon farming through the use of lumpfish as cleaner fish and to minimise the risk of *Kudoa*-infected fish products entering the market place.

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